REMARKS

By the foregoing amendments, claims 77, 78, 80-84, 114-116, and new claims 117 and 118 are pending in this application. Claims 1-76, 79, and 85-113 have been canceled, and claims 80-82 have been withdrawn.

Independent claim 77 has been amended to clarify that the recited "control sample level" of expression is from cells that are not prostate tumor cells. For example, the control level may be expressed from non-tumor cells of (i) the patient (*e.g.*, prostate cells from the patient at an earlier stage in the patient's life or other tissue cells of the patient in which HML-2 expression products are not up-regulated), (ii) a different individual, or (iii) pooled normals. See page 24, line 7 to page 25, line 16 of the specification. Claim 77 has been further amended to recite that the expression product is an RNA comprising a Gag or Pol encoding sequence of the retrovirus. See claims 4, 7 and 9 as originally filed, page 16, lines 5-12 of the specification, in addition to page 77, lines 4-12, page 77 line 28 to page 78 line 8 of the specification and Table 6, identifying the 16 clones of prostate cancer mRNA expression products of HERV-K(CH) as being from the Gag or Pol encoding region of this retrovirus.

Claim 83 has been amended to recite that the expression product is an RNA comprising a Pol encoding sequence of HERV-K(CH), and new claim 117 has been added to recite that the expression product is an RNA comprising a Gag encoding sequence of HERV-K(CH). Accordingly, amended claim 84 (dependent on claim 83) and new claim 118 (dependent on claim 117) recite SEQ ID NOS corresponding to Gag and Pol encoding sequences of HERV-K(CH), respectively.

The claim amendments and new claims add no new matter.

January 12, 2010 Examiner Interview

The undersigned is thankful the courtesies extended during the January 12, 2010 phone interview with Examiner Humphrey. As discussed, independent claim 77 has been amended to better define the control level of expression and recite that the expression product is an RNA comprising a Gag or Pol encoding sequence of HERV-K(CH).

Rejection under 35 U.S.C. § 112, ¶ 1 (Enablement)

Claims 77, 78, 83, 84, and 114-116 have been rejected under 35 U.S.C. § 112, ¶ 1 as not enabled. Applicants respectfully traverse this rejection insofar as it applies to these claims as now amended, in addition to new claims 117 and 118.

To advance prosecution, independent claim 77 has been amended, as suggested by the Examiner, to recite that the expression product is an RNA comprising a Gag or Pol encoding sequence of HERV-K(CH). As noted above, the 16 clones of prostate cancer mRNA expression products of HERV-K(CH), identified in Table 6 as being up-regulated in prostate cancer patients, are from a Gag or Pol encoding region of this retrovirus. The subject matter of claim 77 and its dependent claims 78, 83, 84, and 114-118 therefore more closely conforms to the experimental data in Applicants' specification, with respect to the particular retrovirus tested and particular expression products observed.

The Office Action on page 4 questions whether the up-regulation of mRNA expression products in prostate tissue would correlate to a finding of these expression products in blood. In response, Applicants respectfully refer to evidence in the literature of metastatic spread observed in cancer patients and found to result in the presence of prostate tumor cells in the circulation. The expression products of these cancer cells are detectable using methods such as reverse transcriptase-polymerase chain reaction (RT-PCR). See, for example, Jaakkola *et al.*, CLIN.

CHEM., (1995) 41(2): 182-186 ("Jaakkola"). As explained in the Abstract, "Expression of mRNA specific for a certain tissue in peripheral blood is thought to indicate the presence of circulating cancer cells and metastatic spread of a tumor originating from this tissue." Using RT-PCR to detect the expression of prostate-specific antigen (PSA) mRNA, Jaakkola concludes that patients with prostate cancer have prostatic cells in peripheral blood. See page 185, left-hand column, lines 30-32 of Jaakkola. According to Jaakkola, "each prostate cancer cell may be expected to contain so many copies (5,000-50,000) of PSA mRNA that even 1% of the RNA obtained would be likely to contain enough PSA mRNA to be detected by RT-PCR." See page 185, right-hand column, lines 27-31 of Jaakkola. Thus, the presence of prostate tumor cells in the blood enables the detection of their mRNA expression products, such as PSA, in the blood. One would therefore reasonably expect that other prostate-specific mRNA expression products, including those of HERV-K(CH), could be detected in blood using such well established assays, to the same extent as detected in the prostate tissue itself.

The Office Action also asserts that "it is unpredictable" whether the observed expression products are definitively indicative of prostate cancer but not, for example, breast cancer. See the sentence bridging pages 4-5 of the Office Action. However, the first row of Table 10 on page 92 of the specification shows that 65% of the prostate tissue samples from patients showed up-regulation of sequences in GenBank ID/Accession AB047240, while 0% of breast tissue samples and 2% of colon tissue samples showed up-regulation of these sequences. Importantly, the GenBank ID/Accession AB047240, corresponding to these observed results in the first row of Table 10, were obtained from the 16 mRNA clones of HERV-K(CH) that (i) are referenced in Tables 4-7 as well as Figure 1 of the specification and (ii) have sequences described in Table 8 of the specification.

Evidence of this association between the GenBank ID/Accession AB047240 and the 16 mRNA clones of HERV-K(CH) is found in the specification. See page 37 lines 20-21, page 76 line 28 to page 77 line 14, page 78 lines 9-17 and Table 5 on page 87 of the specification. The nature of this evidence is specifically described in paragraph 6 of the accompanying Declaration of Pablo D. Garcia under 37 C.F.R. § 1.132 ("Declaration"). Paragraph 7 of the Declaration refers to Exhibits B and C, which confirm that GenBank ID/Accession AB047240, referred to in Table 10, corresponds to the 16 mRNA clones of HERV-K(CH) noted above. Based on the experimental results described in the Declaration and shown in Table 10 of the specification, HERV-K(CH) expression products are up-regulated in tissue from prostate tumors, but not in tissue from colon or breast tumors.

With respect to the Depil, Herbst, and Willer papers cited on page 4 of the Office Action, Applicants do not dispute the findings that HERV-K gene products (*e.g.*, HERV-K 10 transcripts) have been detected in various tissue samples of patients afflicted with breast cancer, leukemia, *etc.* The question of enablement, however, must be analyzed in view of the breadth of the claims, as acknowledged by the Office Action's citation on page 3 to *In re Wands* (factor #8). The claims have been limited, to overcome previous rejections based on enablement, to the particular HML-2 retrovirus that is HERV-K(CH). There is nothing in the Depil, Herbst, or Willer papers indicating that the gene products of this particular HML-2 retrovirus are up-regulated in response to disease states other than prostate cancer. As noted above, the highly prostate-specific expression of HERV-K(CH) is confirmed through the data provided in Table 10 of Applicants' specification.

Finally, Applicants respectfully note that absolute demonstrations of therapeutic success are not required to satisfy the enablement standard under 35 U.S.C. § 112, first paragraph. *In re*

Brana, 51 F.3d. 1560, 1568 (Fed. Cir. 1995). The pending claims are claiming a screening method, not a method requiring absolute predictability in terms of a correlation between up-regulation of HERV-K(CH) expression products and prostate cancer. Indeed, cancer diagnostics and therapeutics need not be 100% effective to be recognized as significant contributions to the art. Herceptin®, a therapeutic only effective against 20-30% of breast cancers, is a prime example. In the case of the presently claimed screening methods, even assuming for the sake of argument that an expression product may be detected due to a condition other than prostate cancer, this only indicates that further testing such as a biopsy should be conducted to actually confirm a diagnosis prostate cancer. On this point, Jaakkola notes that a negative result in a patient blood sample was always obtained in healthy controls, patients with benign prostatic hyperplasia (BPH), and patients with other types of cancer. See the Abstract of Jaakkola. Likewise, according to the claimed methods, significant value is derived from the ability to easily screen the vast majority of patients who will not exhibit up-regulation of the expression products being detected.

Please reconsider and withdraw the rejections under 35 U.S.C. § 112, ¶ 1.

CONCLUSION

In view of the above amendments and remarks, all pending claims of this application are believed to be in condition for allowance. A written indication of the same is respectfully requested. This response is believed to completely address all of the substantive issues raised in the final Office Action mailed August 20, 2009.

Please continue to direct all correspondence in this application to Novartis Vaccines and Diagnostics, Inc. (formerly Chiron Corporation), at the address provided for Customer No. 27476.

Respectfully submitted,
BANNER & WITCOFF, LTD.

Date: January 20, 2010

By: _____
Benjamin C. Spehlmann
Reg. No. 45,649

Customer No. 22907

CLIN. CHEM. 41/2, 182–186 (1995) • Molecular Pathology

Detection of Prostatic Cells in Peripheral Blood: Correlation with Serum Concentrations of Prostate-Specific Antigen

Susanna Jaakkola,[1,3] Timo Vornanen,[2] Jari Leinonen,[1] Sakari Rannikko,[2] and Ulf-Håkan Stenman[1]

We have studied the expression of prostate-specific antigen (PSA) mRNA by reverse transcriptase–polymerase chain reaction in peripheral blood of 25 patients with cancer of the prostate (CAP), four with benign prostatic hyperplasia (BPH), two with renal stones, three with other types of cancer, and six healthy male and three female controls. Expression of mRNA specific for a certain tissue in peripheral blood is thought to indicate the presence of circulating cancer cells and metastatic spread of a tumor originating from this tissue. We detected PSA mRNA in 9 of 18 CAP patients with metastatic disease but in none of 7 patients without metastases. Negative results in patients with metastatic disease were associated with successful endocrine therapy and low concentrations of serum PSA, and the correlation between serum concentrations of PSA and the presence of PSA mRNA in peripheral blood was statistically significant. PSA mRNA was not found in patients with BPH, other types of cancer, or in healthy controls. Thus the occurrence of PSA mRNA in peripheral blood is associated with metastatic CAP.

Indexing Terms: *mRNA/reverse transcriptase/polymerase chain reaction/prostate cancer/metastases*

Metastatic spread of prostatic tumors can be both lymphatic and hematogenous. Hematogenous spread occurs overwhelmingly to the bones and less so to the lungs and liver (*1, 2*). Hematogenous dissemination of cancer is generally thought to be an inefficient process. Animal experiments suggest that only ~0.01% of circulating cancer cells actually form metastases (*3–5*). Thus a concentration of two cancer cells per milliliter of blood could be sufficient for hematogenous tumor spread (*6*), but detection of these cells has been difficult because of lack of sufficiently sensitive techniques. Immunocytological methods have been used successfully to identify neuroblastoma cells in peripheral blood. By this method one tumor cell can be found among 10^5 normal nucleated blood cells (*7*). However, recent studies suggest that by reverse transcriptase–polymerase chain reaction (RT-PCR) it is now possible to detect a single neuroblastoma (*8*) or melanoma cell (*9*) in a 2-mL blood sample corresponding to ~10^7 nucleated cells.[4]

Demonstration of circulating prostate cancer cells in patients with cancer of the prostate (CAP) may be a sensitive method to identify metastasizing tumors or new cancer cases. Flow cytometry (*10*) and recently detection of prostate-specific antigen (PSA) mRNA by RT-PCR have been used for this purpose (*6*). We have evaluated the usefulness of RT-PCR for detection of prostatic cells in peripheral blood of patients with prostatic cancer and hyperplasia and compared the results with the serum concentrations of PSA.

Materials and Methods

Patients

We studied 25 patients with CAP (Table 1), four with benign prostatic hyperplasia (BPH), two with renal stones, and three with other cancers (one renal cancer, one testicular cancer, and one bladder cancer). Three healthy women and six healthy men were also studied (Table 1). All the CAP patients had been treated either by surgery or by hormone therapy, and 18 of them had bone metastases as indicated by isotope scanning. Blood samples were collected in 5- or 10-mL Vacutainer EDTA tubes (Becton Dickinson, Rutherford, NJ). The diagnoses of CAP and BPH were verified by histology of surgically removed tissue. In all but one patient the blood samples from the CAP patients were drawn 1 month to 2 years after surgical therapy. All but two patients either received hormonal therapy or had been orchiectomized (Table 1). In BPH patients, blood samples were obtained before transurethral resection of the prostate (TURP). One of these proved to have an incidental CAP and was included in the CAP group.

Tissue Samples

Prostatic tissue removed during TURP was used as a positive control for PSA mRNA.

Isolation of Nucleated Cells and RNA Extraction

Red blood cells were hemolyzed by adding 1.5 volumes of diethylpyrocarbonate-treated water to 1.0 vol-

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[4] Nonstandard abbreviations: PSA, prostate-specific antigen; RT-PCR, reverse transcriptase–polymerase chain reaction; CAP, cancer of the prostate; BPH, benign prostatic hyperplasia; TURP, transurethral resection of prostate; and hGK-1, human glandular kallikrein; AMV; avian myeloblastosis virus; and EMBL, European Molecular Biology Laboratory.

Table 1. Clinical characteristics, PSA mRNA, serum PSA concentrations, and treatment of patients with prostate cancer and controls.

Age, years	PSA mRNA	PSA, mg/L	Stage	Grade	Bone scan	Treatment[a]
Prostate cancer patients						
78	+	0.5	T2	G3	+	E
80	+	42.0	T3	G2	+	Orch.
66	+	85.0	T3	G2	+	Pros., orch., E
69	+	99.0	T4	G3	+	TURP, Zoladex
73	+	143.0	T4	G2	+	Orch., TURP
78	+	317.0	T3	G2	+	Pros., orch., E
81	+	846.0	T3	G2	+	Orch.
62	+	289.0	T2	G3	+	Pros., orch.
64	+	1403.0	nd	G3	+	E
57	–	0.5	nd	G3	+	Orch.
85	–	495.0	T3	G2	+	TURP
62	–	1180.0	T3	G2	+	Orch.
62	–	0.5	nd	G3	+	Orch.
71	–	74.0	nd	G2	+	Orch.
75	–	2.7	nd	G3	+	E
69	–	1.0	nd	nd	+	Orch., E
67	–	36.0	nd	G3	+	Zoladex
65	–	nd	nd	nd	+	Flutamide
81	–	3.3	T1b	G3	–	TURP, orch.
79	–	47.0	T4	G1	–	Orch.
81	–	0.5	T3	G1	–	Orch., TURP
66	–	nd	T3	G1	–	Pros.
57	–	17.0	nd	G2	–	E, Zoladex
74	–	0.5	nd	G3	–	Zoladex
73	–	0.5	T1b	G1	–	TURP

		PSA, μg/L	Diagnosis		
Controls					
Benign urological diseases					
59	–	nd	BPH	nd	TURP
86	–	13.4	BPH	nd	TURP
72	–	1.3	BPH	nd	TURP
72	–	1.7	BPH	nd	nd
32	–	nd	Renal stone	nd	nd
65	–	nd	Renal stone	nd	nd
Other cancers					
67	–	nd	Renal cancer	–	Nephrectomy
25	–	nd	Testicular cancer	–	Pros., orch.
75	–	nd	Bladder cancer	–	nd
Other controls, men					
22	–	nd			
29	–	nd			
29	–	nd			
32	–	nd			
37	–	nd			
27	–	nd			
Controls, women					
27	–	nd			
30	–	nd			
33	–	nd			

nd, not done.
[a] Orch., orchiectomy; pros., prostatectomy; E, estrogen.

ume of blood. After 5 min the nucleated cells were collected by centrifugation. Total RNA was extracted from both nucleated blood cells and prostatic tissue by the guanidinium thiocyanate/phenol/chloroform method (*11*). Heparinized blood was not used because heparin has been reported to inhibit both murine leukemia virus reverse transcriptase and Taq DNA polymerase (*12*). Total RNA integrity was checked by formaldehyde gel electrophoresis. In addition, RT-PCR with specific β-globin control primers was used for blood samples.

Oligonucleotide Primers

Specific oligonucleotide primers for human PSA were designed on the basis of sequence data from the European Molecular Biology Laboratory (EMBL) gene bank. The primer pairs were selected to locate in different exons, so that the possible PCR product from genomic DNA could be distinguished from that obtained from cDNA. The primers were located in exons 3 (PSA outer sense), 4 (PSA nested sense), and 5 (PSA nested antisense and outer antisense) of the PSA gene. The outer primers produce a PCR fragment of 508 bp and the nested primers a fragment of 194 bp (Fig. 1). PSA displays a high degree of homology with the human kallikreins and, like the PSA gene, the human glandular kallikrein (hGK-1) gene is expressed in the human prostate (*13–15*). All four PSA primers were aligned with the hGK-1 sequence to avoid amplification of hGK mRNA. The sequences of the PSA primers are shown in Table 2.

β-Globin primers were devised on the basis of the published sequence (*16*). They are located on either side of intron 2 and produce a fragment of 283 bp. The sequences for β-globin primers were: Glo sense: ACC CAG AGG TTC TTT GCG TC; Glo antisense: TCT GAT AGG CAG CCT GCA CT.

RT-PCR

Of total RNA, 10–50 μg was usually isolated from 5–10 mL of blood. For reverse transcription, 1 μg of this RNA was incubated in a 20-μL reaction mixture con-

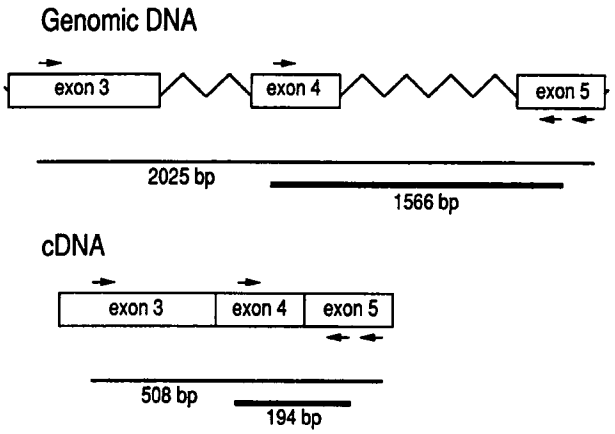

Fig. 1. Physical locations of PSA primers in genomic and cDNA and sizes of various PCR products.

Table 2. Sequences for PSA oligonucleotide primers.

Primer	Sequence	Bases
Outer sense	CAC AGG CCA GGT ATT TCA GG	560–579
Outer antisense	CCT TGA TCC ACT TCC GGT AA	1049–1068
Nested sense	TCC AAT GAC GTG TGT GCG CA	842–861
Nested antisense	GTG TAC AGG GAA GGC CTT TC	1017–1036

sisting of 1× RT buffer (50 mmol/L Tris-HCl, pH 8.3, 50 mmol/L KCl, 10 mmol/L $MgCl_2$, 10 mmol/L dithiothreitol, 0.5 mmol/L spermidine), 20 U of RNasin (Promega, Madison, WI), 1 mmol/L of each dNTP (dATP, dCTP, dGTP, dTTP), and 80 pmol of the outer antisense primer. After denaturation for 5 min at 65°C, 10 U of avian myeloblastosis virus (AMV) RT (Promega) was added and the samples were incubated at 42°C for 1 h and at 52°C for 30 min. The possible contamination of the RNA samples was excluded by subjecting each sample to RT without AMV RT before PCR. For the PCR reaction the samples were diluted 50-fold in TE (10 mmol/L Tris-HCl, pH 8.0, 1 mmol/L EDTA), and 5 μL was amplified in a 50-μL reaction volume in 1× PCR buffer (10× buffer consisted of 10 mmol/L Tris-HCl, pH 8.8, 1.5 mmol/L $MgCl_2$, 50 mmol/L KCl, 1 mL/L Triton X-100; Finnzymes, Espoo, Finland), 0.2 mmol/L of each dNTP, 20 pmol of both antisense and sense primers, and 1.25 U of Dynazyme DNA polymerase (Finnzymes) for 30 cycles at 95°C for 40 s, 65°C for 60 s, and 72°C for 90 s. Alternatively, we used 30 cycles at 95°C for 45 s and 65°C for 45 s. For reamplification, 5 μL (1/10) of the first PCR product was further amplified with the nested primers in a 50-μL reaction volume for 30 cycles. Ten microliters of the product was electrophoresed on a 1.5% agarose gel and visualized by ethidium bromide staining. RNA isolated from prostatic tissue was used as a positive control and water as a negative control in all experiments.

Restriction Enzyme Analysis, Cloning, and Sequencing of the PCR Product

The identity of the PCR product was confirmed with *Ava*I restriction enzyme digestion and sequencing of the product. This enzyme has one restriction site within the 194-bp PCR product. For sequencing, the 194-bp product generated from PSA cDNA with nested PCR primers was cloned into a pCRII plasmid vector (TA Cloning Version 1.3; Invitrogen, San Diego, CA) as described by the manufacturer. Of this plasmid DNA, 4 μg was sequenced with the nested sense PSA primer with Version 2.0 DNA Sequencing Kit from United States Biochemical (Cleveland, OH).

Southern Transfer Analysis

A 1.5% agarose gel containing 16 negative samples and 1 positive control sample was blotted onto a Hybond-N (Amersham Intl., Amersham, UK) nylon membrane according to the manufacturer's instructions. The hybridization was carried out at 65°C overnight in hybridization solution containing 1 mol/L NaCl, 100 g/L dextran sulfate, and 10 mL/L sodium dodecyl sulfate. The membrane was washed under high stringency conditions. The sequenced PCR product, labeled by nick translation, was used as a probe.

Detection Limit

The detection limit of the method was established by adding LNCap cells, a prostate carcinoma cell line expressing PSA, to blood samples. LNCap cells were cultured in RPMI 1640 medium supplemented with fetal calf serum (100 mL/L), glutamine (2 mmol/L), penicillin (100 kU/L), streptomycin (100 mg/L), and Fungizone (amphotericin B; 2.5 mg/L). Cells were transferred from culture bottles with 2.5 mL/L trypsin. The leukocytes in the blood samples and the LNCap cells were counted, and dilutions of LNCap cells were mixed with blood to give 1–1000 LNCap cells per 6×10^9 leukocytes per liter. Total RNA was isolated and RT-PCR was done as described above.

Statistical Analysis

The difference in serum PSA concentrations between patients who tested positive or negative for PSA mRNA was analyzed by Student's *t*-test after logarithmic transformation of the PSA values.

The study protocol was in accordance with Helsinki Declaration of 1975, as revised 1983.

Results

The expected 508-bp PSA band was readily detected after 30 cycles with the outer primers in RNA from prostatic tissue, but this band was not detected in blood samples. The 194-bp PSA band was detected after 30 cycles with the outer and 30 with the nested primers (Fig. 2). Contamination of the RNA preparation with genomic DNA is a potential problem. When the PCR cycling consisted of incubations at 95°C for 40 s, 65°C for 60 s, and 72°C for 90 s, genomic DNA was occasionally amplified, giving a single 1566-bp band. Thus it was readily distinguished from the RNA-derived band. The genomic band could be eliminated by using shorter cycles, i.e., 95°C for 45 s and 65°C for 45 s. Amplification with the β-globin primers showed that the isolated mRNA was intact (Fig. 3).

Amplification of the PSA rather than the hGK-1 gene was ascertained by restriction enzyme analysis and by sequencing the PCR product and comparing the sequence to the PSA and human kallikrein sequences in

Fig. 2. RT-PCR with outer and nested PSA primers.
The product of outer primers (508 bp) can be detected in prostatic tissue after 30 cycles (*lane 4*), but in blood samples the 194-bp band of PSA was detected only by using 2 × 30 cycles of PCR with outer and nested primers (*lanes 1–3*). *Lanes 5* and *6*, negative controls. M, λ/*Hin*dIII and φX174/*Hae*III markers.

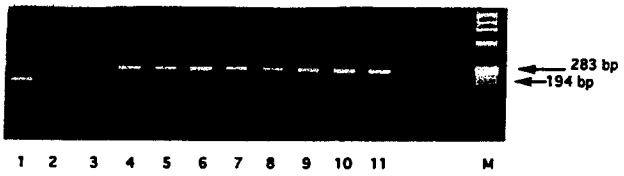

Fig. 3. Control RT-PCR with β-globin primers (283 bp) of eight samples (*lanes 4–11*).

Lanes 2 and *3*, negative controls. *Lane 1*, RT-PCR with nested PSA primers (194 bp) of prostatic tissue. M, λ/*Hin*dIII and φX174/*Hae*III markers.

the EMBL gene bank. The product had the expected sequence. All the negative samples were also negative by Southern transfer analysis, which has a detection limit below that of ethidium bromide staining.

PSA mRNA was detected in blood samples from 9 of 18 CAP patients with bone metastases. All these patients had moderately or poorly differentiated (grade 2, seven cases, and grade 3, two cases) cancer. PSA mRNA was detectable neither in blood from nine other patients with a positive bone scan nor from patients without metastases, four of which were well differentiated (grade 1). All patients with BPH as well as healthy controls with benign disease and other types of cancers were also negative (Table 1).

When LNCap cells were added to normal blood samples, PSA mRNA was detected by RT-PCR in samples containing 10–1000 LNCap cells per 1 mL of blood. Thus LNCap cells could be detected at a concentration of 1.6 cells/10^6 blood leukocytes.

Because expression of PSA mRNA may be expected to correlate with expression of PSA, we compared the serum concentrations of PSA with the results for PSA mRNA. The concentrations were significantly higher ($P = 0.02$) in patients with detectable mRNA, but there was some overlapping. Thus one patient with a PSA concentration <1 μg/L tested positive, whereas two patients with PSA concentrations of 495 and 1180 μg/L tested negative for PSA mRNA (Table 1).

Discussion

The results of this study confirm that many patients with prostate cancer have prostatic cells in peripheral blood. However, in half of the patients with advanced cancer treated by castration or with estrogens, PSA mRNA could not be detected. Similar results have recently been reported in studies based on flow cytometry and antibodies to PSA (*10*) and by RT-PCR with different sets of primers (*6*). By flow cytometry, 83% of the patients tested positive (*10*), as compared with 33% by RT-PCR (*6*). The high proportion of positive cases in flow cytometry was thought to be due to uptake of PSA antigen by host immunocytes rather than expression of PSA mRNA by circulating prostatic epithelial cells (*10*). Thus RT-PCR probably is a more specific method for detecting prostatic cells in blood than is flow cytometry with PSA antibodies. PSA mRNA was detected neither in blood from patients without bone metastases nor in the controls. A negative bone scan does not exclude metastatic disease; thus the number of patients with widespread disease may be larger than indicated in Table 1.

Thirty PCR cycles with the outer PSA primers gave a 508-bp band from prostatic tissue but not from blood samples. Thirty additional cycles with nested primers giving the 194-bp band were needed to detect PSA from blood samples because of the low number of PSA-expressing cells in circulation as compared with prostatic tissue. The negative results in cases with bone metastases suggest an absence of prostatic cells in the blood sample obtained, even though a small number of such cells were present in the circulation. A 5-mL sample represents ~0.1% of the blood volume. Thus, with 1000 cancer cells in the circulation there would be a 70% chance of getting a positive result (according to the Poisson distribution) if the method is able to detect one cell.

By the present method we detected 10 LNCap cells/6 × 10^6 blood leukocytes, corresponding to 1.6 LNCap cells/10^6 blood cells. A recently described RT-PCR had similar sensitivity, i.e., 1 LNCap cell in 10^6 peripheral blood mononuclear cells (*17*).

The sensitivity could be increased by taking a larger volume of blood, but the possibilities of this approach are clearly limited. We used only part (2–10%) of the RNA isolated for the RT reaction, and sensitivity could possibly be improved by using more RNA. However, each prostate cancer cell may be expected to contain so many copies (5000–50 000) of PSA mRNA (*18*) that even 1% of the RNA obtained would be likely to contain enough PSA mRNA to be detected by RT-PCR. Another possible explanation for the negative results is that circulating CAP cells may be poorly differentiated, expressing low concentrations of PSA (*6*). In cancer patients with high serum PSA concentrations this would imply the existence of different cancer cell populations, only some of which express PSA. This is a potential limitation of the present method.

Growth of both normal and malignant prostatic cells is stimulated by androgens. Thus androgen deprivation is commonly used for treatment of CAP, and the response to treatment can be monitored by measurement of PSA in serum (*19*). In CAP cells in culture, the expression of PSA and PSA mRNA is induced by androgens (*20*). Therefore the endocrine treatment used in most of our CAP patients may be expected to suppress expression of both PSA protein and mRNA. In seven patients serum PSA was suppressed to basal concentrations (≤1 μg/L), and in most of these, PSA mRNA was undetectable in peripheral blood. However, in one patient treated with estrogen who had a PSA concentration <1 μg/L, PSA mRNA was detected. This indicates that cells expressing PSA mRNA may occur in circulation, although PSA expression at the protein level may be very low. In two patients with metastatic disease, PSA mRNA was not detected despite very high serum PSA concentrations, 495 and 1180 μg/L. This suggests that high PSA production need not be associated with circulating cancer cells. It remains to be shown whether this finding has any implications for

the progression of the disease. PSA immunoreactivity in breast cancer cytosols has been detected recently, although PSA expression was extremely low (*21*). Therefore, although possible, it is not very likely that the RT-PCR method described here would be useful for detection of circulating breast cancer cells.

This research was supported by grants from The Finnish Cancer Foundation, The Sigrid Juselius Foundation, The Finnish Academy of Sciences, and The Alfred Kordelin Foundation.

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